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RESEARCH ARTICLE

Diversity of ectomycorrhizal hypogeous fungi in the protected area "Tulovska koria"

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Abstract

The paper presents results of an assessment of subterranean fungal diversity in the protected area "Tulovska koria", an endangered forest ecosystem in Bulgaria. High diversity was documented on a relatively small area, consisting of 14 species belonging to 7 families and two orders. Half of the established species belonged to the genus *Tuber* (fam. Tuberaceae). Two of the species are of conservation importance and are therefore included in the Red Data Book of Bulgaria. Nine species were confirmed as a part of the natural Bulgarian mycota.

Keywords

ecosystems, ectomycorrhiza, fungi, diversity, protected areas

Introduction

The protected area „Tulovska koria" is an old-growth forest dominated by the Common oak (*Quercus robur* L.), pedunculate oak (*Quercus pedunculiflora* L.) and European hornbeam (*Carpinus betulus* L.). Initially, it was declared as a natural landmark in 1961 (Anonymous, 1962) and later – as protected area (Anonymous, 2002), corresponding roughly to the category VI of the IUCN classification – protected area with sustainable use of natural resources. Large part of the forest stand here is subjected to substantial pressure and is undergoing a process of negative succession, in which the pedunculate oak is almost completely replaced by the European hornbeam (Zlatanov, 2007). It should be emphasized that the forest ecosystem here possesses some characteristics substantially different from these of a typical old-growth forests, which are characterized by old trees and spatial heterogeneity (Spies, 2004). According to Hilbert

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and Wiensczyk (2007), the old-growth forests can be understood as unmanaged forests that have reached their peak development with zero annual growth, and where the primary tree vegetation is in the stage of its replacement by a secondary one. However, in the site "Tulovska koria" this process has been greatly enhanced by the negative human influence. From a phytogeographic point of view, PA "Tulovska koria" can be referred to the intrazonal vegetation of Thracian forests of *Quercus pedunculiflora* and *Q. robur* (Dimitrov, Tzonev, 2015). This type of natural habitats are mainly distributed in the Upper Thracian Plain and in the Tundzha hilly country. The species composition of the vegetation is influenced by the climatic features of the transitional – continental climate, the hydrological features (the regime of soil moisture and, in particular, the level of groundwater, influenced by their connection with nearby riverbeds), the mode and duration of flooding, as well as the soil type. It is believed that the Thracian forests of *Quercus pedunculiflora* and *Q. robur* are the driest subtype of lowland riparian forests. In most cases, these are old forests with relatively small area and are surrounded by agricultural land (Dimitrov, Tzonev, 2015). This forest habitat is included in the list of endangered, disappearing or endemic ecosystems in Bulgaria (WWF, 2016). From the point of view of biological diversity, old growth forests, with their specific structure and functions, are the habitat of a complex of species of different ecological and taxonomic groups. In this regard, the diversity of ectomycorrhizae in these ecosystems is essential for their adaptation to environmental changes. Due to the limited knowledge about this group of organisms, it is not yet possible to determine how many of the studied species are associated only with these forests, but it can be assumed that many species find here optimal conditions for existence.

In the ectomycorrhizal symbiosis, the plants provide the fungal partner with photosynthesized sugars in exchange for water, minerals, and nutrients such as nitrogen and phosphorus (Smith, Read, 2008). The effect of ectomycorrhiza on plant communities can be considered in two aspects – as biomass accumulation and as positive effects in relation to increasing the general health status of the individual and the plant community (Nedelin, 2017). Similar to phytocoenoses, ectomycorrhizal fungi form mycocenotic communities that are extremely dynamic in time and space, and this is mostly due to the short life span of ectomycorrhizal roots (Downes et al., 1992). Underground (hypogean) species of ectomycorrhizal fungi are, for obvious reasons, one of the least studied part in mycocenotic communities. Their exact ecological role is difficult to determine. A key function of subterranean fungi is the role their fruiting bodies play as a food resource for a large number of terrestrial mammals. For some animals, these fungi form the most important nutritional element throughout the year, while for others they may have only seasonal or supplementary value (Claridge, 2002). Mycophagy can be considered to contribute to the nutrition of small forest mammals and plays a key role in the regeneration and functioning of forest ecosystems by providing mycorrhizal inoculum (Schickmann et al., 2012). In this regard, it can be assumed that the biological diversity of the underground ectomycorrhiza mycota is essential

for the functioning of this type of forest ecosystem as a whole. Their identification is critical to understanding the ecology and conservation of rare and threatened plants, fungi and habitats (Janowski, Leski, 2023). It is believed that the soil conditions and the species composition of the dendroflora have a key role in the biological diversity of the ectomycorrhizal mycota (Bruns, 1995).

Therefore, the objective of the present study was to make a survey on the ectomycorrhizal hypogeous fungi in the protected area "Tulovska koria". Elucidating this aspect of biuodiversity could help the better understanding of the forest structure and function and could bring insight for designing the proper measures for conservation of this valuable and fragile ecosystem.

Materials and methods

Protected area „Tulovska koria" is situated in the Kazanlak valley – Southern Bulgaria, on an area of 75.7 hectares, and approximate coordinates 42°33'49"N 25°33'10"E. The purpose of this protected area is the preservation of remnants of an indigenous oak forest (Anonymous, 1962).

The study was conducted in the periods May – December in 2017 and 2018. Collecting of hypogeous fungi was performed by patrolling the site on random routes (Fig. 1) with the help of specially trained dogs. Species identification followed Montecchini and Sarasini (2000), Pegler et al. (1993) and Riousset et al. (2012). It was done by means of laboratory microscopic examinations, performed at the University of Forestry, Sofia in May 2019. A binocular stereomicroscope with built-in digital camera LEICA EZ4D (8x-35x) was used for the examination as well as laboratory phase contrast trinocular microscope LEICA Laborlux S with magnification 100x-2000x (over 800x with immersion). The specimens collected in the field were dried and put in a specialized collection.

The decolorizing or coloring agents used to better visualize fungal structures were the following ones: 3-5% KOH in distilled or tap water (H_2O), Congo red at 10% rpm ammonia (CR); Cotton Blue (CB) in lactophenol and Melzer's reagent (MR). KOH gives a good idea of the size of certain fungal structures and has a lightening effect. CB and MR stain the fungal structures in blue and yellow, respectively, with the former yielding better contrast than the latter.

The presentation of each species recorded in the locality follows the mode: brief decription of the species, and its seasonality, meaning the period when the species had been established in the area.

Results and discussion

Total fourteen hypogeous taxa of fungi, belonging to 7 families and two orders, were established in the region of study – protected area "Tulovska acoria". Eleven species

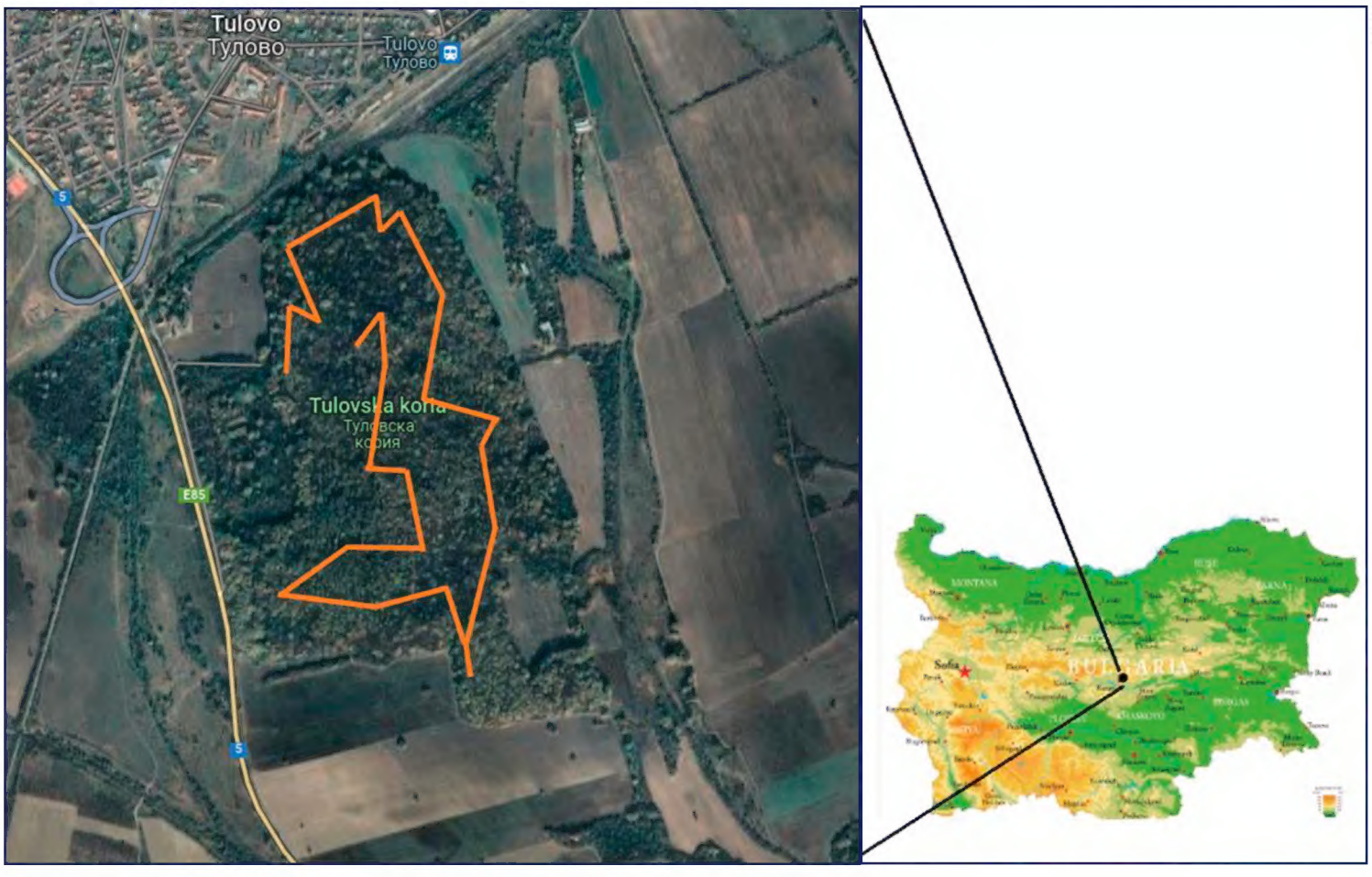

Figure 1. Situation and map of the region of study. The orange line indicates the route where the inventory of hypogeous fungi took place.

belonged to the order Ascomycota and three species to the order Basidiomycota. Seven species belonged to family Tuberaceae, two – to fam. Pyronemataceae and the families Elaphomycetaceae, Hymenogasteraceae, Paxillaceae, Boletaceae and Pezizaceae were represented by one species each. The details about the recorded taxa are presented in Table 1. The photograph of the spores of identified taxa are presented on Fig. 2.

Below we present more detailed information about the different taxa found in the protected area.

Elaphomyces papillatus Vittad.

This species is a relatively small truffle having a specific texture (leathery-rough) on the surface of the fruiting bodies and coloring of the peridia. It is called deer truffle and is characterized by a strong thyme-like aroma. Besides, it is one of the few of its type occurring also in alkaline soils (Paz et al., 2017). It was found on a such soil type in the largest park of Sofia city – "Borissova gradina". This species was reported for Bulgaria for the first time by Nedelin et al. (2018).

Seasonality: Only one fruiting body was found in August 2018 in a group of older hornbeam trees (Table 1). In Sofia, it was found in various places, but with single fruiting bodies, during the IV-VI period.

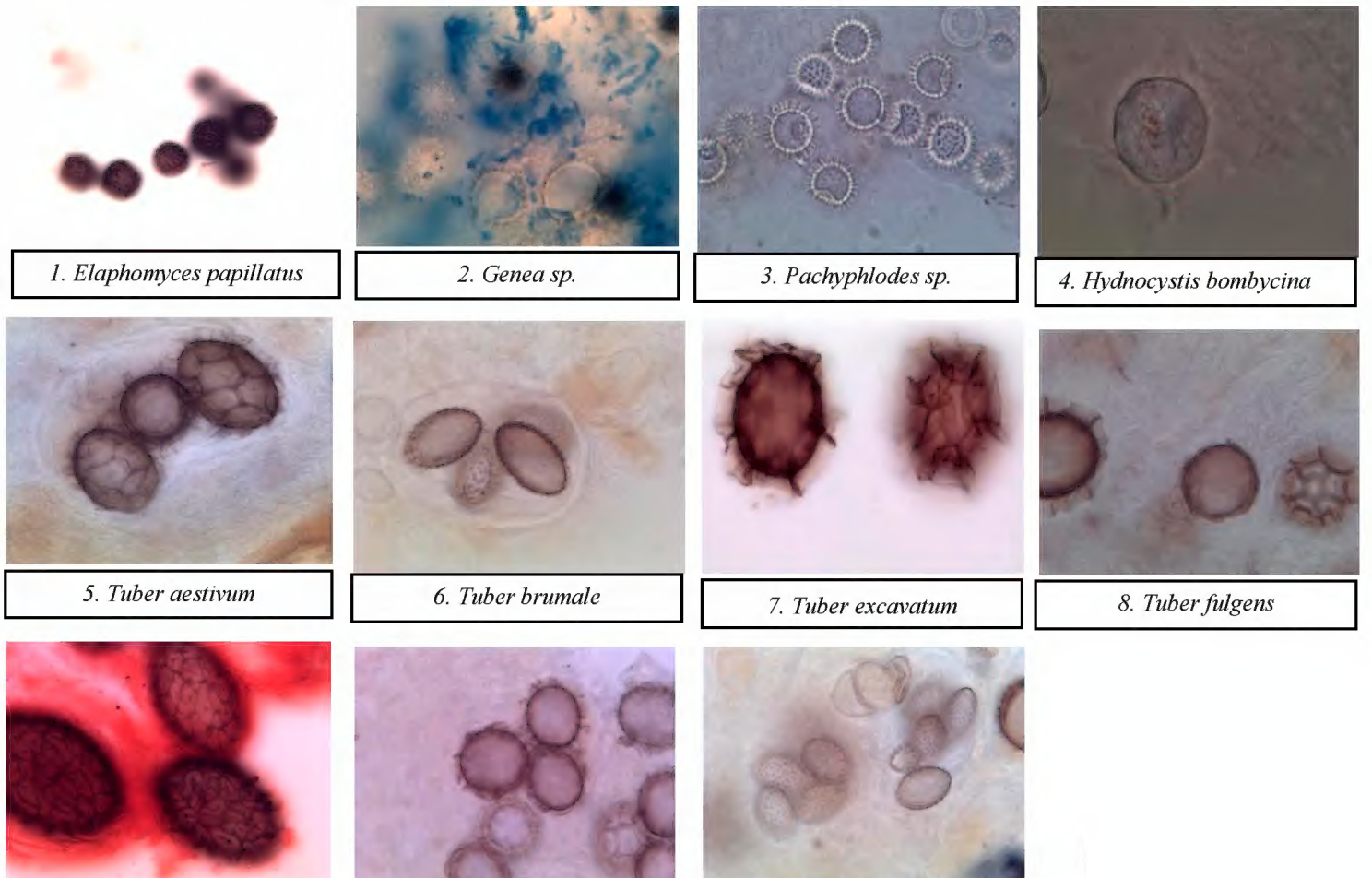

Figure 2. Spores of the species, recorded in PA "Tulovska koria".

Genea sp.

The species of genus *Genea* are very difficult to identify, even by microscopic characters (Fig. 2). Using the most comprehensive study in this respect, the monograph of Alvarado et al. (2014) we came to conclusion that one-recorded species, numbered as 32, seems very close to *G. pseudoverrucosa*; however, molecular testing is needed for confirmation. So far, only *G. pseudoverrucosa* and *G. arenaria* (Assyov and Slavova, 2017) have been reported for Bulgaria.

Seasonality : Only one fruiting body was found in December 2018 in a group of old oak trees (Table 1).

Pachyphlodes sp.

The genus *Pachyphlodes* was first registered in Bulgaria by Assyov and Slavova (2017). It can be easily distinguished by its small size, slightly jelly-like gleba and

Table.1. Identified hypogeous fungi in PA „Tulovska koria“

№	Species	Family	Potential plant host	Period of observation
	Order Ascomycota			
1	*Elaphomyces papillatus* Vittad	Elaphomycetaceae	*Carpinus betulus* L.	VI-IX
2	*Genea* sp.	Pyronemataceae	*Quercus cerris* L. *Quercus robur* L.	
3	*Pachyphlodes* sp.	Pezizaceae	*Quercus cerris* L. *Quercus robur* L.	V-VII
4	*Hydnocystis bombycina* (Vittad.) Healy & M.E. Sm	Pyronemataceae	*Carpinus betulus* L.	V-VII
5	*Tuber aestivum* Vitt.	Tuberaceae	*Carpinus betulus* L. *Tilia* sp. *Quercus cerris* L. *Quercus robur* L. *Corylus avellana* L.	III-XI
6	*Tuber brumale* Vittad.	Tuberaceae	*Quercus robur* L. *Quercus cerris* L. *Populus alba* L. *Tilia* sp.	X-III
7	*Tuber excavatum* sl	Tuberaceae	*Quercus robur* L. *Quercus cerris* L. *Corylus avellana* L. *Populus alba* L. *Salix* sp.	V-XII
8	*Tuber fulgens* Quél	Tuberaceae	*Tilia cordata* Mill.	VIII-XI
9	*Tuber macrosporum* Vittad.	Tuberaceae	*Quercus cerris* L. *Quercus robur* L. *Carpinus betulus* L. *Corylus avellana* L. *Populus alba* L. *Salix* sp.	IX-XII
10	*Tuber magnatum* Picco	Tuberaceae	*Quercus robur* L. *Carpinus betulus* L. *Populus nigra* L. *Populus alba* L. *Salix* sp.	IX-XII
11	*Tuber rufum* Pollini	Tuberaceae	*Quercus robur* L. *Quercus cerris* L. *Carpinus betulus* L. *Corylus avellana* L. *Populus nigra* L. *Populus alba* L.	IV-X
	Order Basidiomycota			
1	*Hymenogaster luteus* Vittad.*	Hymenogasteraceae	*Corylus avellana* L.	XI-II
2	*Melanogaster broomeanus* Berk	Paxillaceae	*Corylus avellana* L.	V-VIII
3	*Octaviania asterosperma* Vittad.*	Boletaceae	*Carpinus betulus* L. *Quercus cerris* L. *Quercus robur* L.	VI-IX

* the species was included in the Red Data Book of Bulgaria (Gyosheva, Stoychev, 2015; Denchev et al., 2015)

“spiny” spores. Its peridium is almost smooth with lateral indentations. According to Montecchi and Sarasini (2000), there are four species occurring in Europe. A key element for species identification is the size of the asci, but no such were noticeable microscopically in the specimens found in the „Tulovska koria“ due to the advanced degree of maturity. Additional studies are necessary for exact species identification, including DNA taxonomic markers.

Seasonality: More than 10 fruiting bodies were found in August 2018 in a group of oak trees.

Hydnocystis bombycina (Vittad.) Healy & M.E. Sm.

The species *Hydnocystis bombycina* was reported for Bulgaria under the name *Stephensia bombycina* by Gyosheva et al. (2012a). It can be easily recognized by its unpleasant smell of rotten eggs. This species was recorded in various habitats in Bulgaria, and has been represented usually by few fruiting bodies.

Seasonality: One fruiting body was recorded in August 2018 in hornbeam understorey.

Tuber aestivum Vitt.

The black summer truffle, also known as the Burgundy truffle, is the most important and the most widely distributed commercial type of truffle in Bulgaria. It was one of the first underground species reported for the country (Hinkova 1961) and was later confirmed by Savev et al. (2006). It is included in the Red Data Book of Bulgaria with the category endangered (EN) (Gyosheva et al. 2006), but according to unofficial data, from one hundred to eight hundred tons are exported annually from Bulgaria. It is easily recognized by the peridia and by its aroma, which is well expressed only in autumn. There is great a diversity within populations. Molecular studies (Zambonelli et al., 2016) showed that *T. aestivum* and *T. uncinatum* Chatin must be classified as the same species.

Seasonality: The species was established in July, September and December 2018, with more than ten fruiting bodies found in different locations in the PA "Tulovska koria", within hornbeam and oak groups of trees.

Tuber brumale Vittad.

Black winter truffle is a commercial species related to different tree species. Several different varieties have been described (Montecchi & Sarasini 2000). This species has needle spores. Its peridium is warty and somewhat similar to the summer black truffle, but it is smoother and with brown-violet coloration observed between the warty outgrowths of the peridium when viewed under a magnifying glass. Measurements of spores from "Tulovska koria" population showed greater elongation than that described by Montecchi and Sarasini (2000). However, the above authors also cite other data – Sarasini and Bincoletto (1997), which indicate similar sizes, with the exception of Q (the ratio length:width), which was within the range 1.4-1.7.

Seasonality : This species occurs from mid-autumn to early spring. It was found in December 2018, with several fruiting bodies recorded, near linden (*Tilia*) trees.

Tuber excavatum Vittad. *s. l.*

Tuber excavatum complex occurs within a range of conditions. The whole group is easily recognized by the hard, almost smooth shell of the peridium and the well-defined cavity. Nedelin et al. (2016) did a survey on all available sequences in the gene

bank at that time and found that intraspecific variability was greater than interspecific one, leading to distinguishing of two groups. This study was also confirmed by other authors – a new species belonging the group was described (*Tuber pulchrosporum*) with two localities – around Sliven (with common oak) and around Varna (with common hornbeam) (Polemis et al., 2019). *T. excavatum s.l.* records had been reported from Northeast Bulgaria (Dimitrova and Gyosheva, 2008) and from Western Rhodopes (Lacheva, 2012).

Seasonality: The species of this group, were found almost all year round. Several fetal bodies were found in September – December 2018.

Tuber fulgens Quél

The species *Tuber fulgens* was reported for the first time for Bulgaria by Assyov and Slavova (2017). In the field, it can be recognized relatively easily by the bright orange color and hollow fruiting body, and microscopically by the round or almost round spores.

Seasonality: Three fruiting bodies were found in December 2018 in a linden group of trees (Table 1).

Tuber macrosporum Vittad.

Tuber macrosporum is a commercial species and is one of the most valuable truffle species. It is recognized by the smooth and cracked peridium, the rich aroma and the spores, which are large and can be seen with the help of a magnifying glass. It was described for Bulgaria by Gyosheva et al. (2012b). According to Montecchi and Sarasini (2000) its habitat is similar to that of the white truffle – *Tuber magnatum.*

Seasonality: It was found several times in the fall of 2018 in the „Tulovska koria" in different groups of trees (Table 1). It is being harvested together with the white Italian and the black summer truffle and is an important commercial species.

Tuber magnatum Picco

The white truffle (*Tuber magnatum)* is the most valuable commercial species. It is easily recognized by its rich and superior aroma. Another important feature is the shape of its spores, which are nearly round with up to 3 pentagons projected into them. In the present study, it was noted in symbiosis with the common hornbeam.

Seasonality : According to literary sources, it occurs only in autumn. One fetal body was found on 11th of November 2017 at a depth of about 30 cm.

Tuber rufum Pollini

This type of truffle is relatively easy to recognize. It is am inedible and relatively common species in Europe, including in Bulgaria. Several varieties have been described (Montecchi and Sarasini, 2000). In Bulgaria it was reported for the first time by Nedelin et al. (2016).

Seasonality: *T. rufum* was found in September 2018. Only a few fruiting bodies were recorded in the PA „Tulovska koria".

Conclusion

The current study of the subterranean mycota in the PA 'Tulovska koria" revealed a great species diversity within a relatively small area. Total 14 subterranean ectomycorrhizal species have been identified. The main tree phytobionts belong to four genera. Half of the established species belong to the genus *Tuber*. The studied forest is one of the few in Bulgaria with so many representatives of the genus. The main problem related to the conservation of the subterranean fungal species is focused to the successful regeneration of the oak. Accordingly, the permanent change in the soil moisture regime in the forest territory poses a threat to the hypogean habitat mycota (Zlatanov, 2007). The climate factor threatens diversity and a number of measures must be taken to reduce its impact.

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